



NATIONAL INSTITUTE OF TECHNOLOGY, ROURKELA

**PRODUCTION AND CHARACTERIZATION OF  
EXTRACELLULAR POLYMERIC SUBSTANCES  
OF *RHIZOBIUM* WITH DIFFERENT CARBON  
SOURCES**

**Dissertation submitted in partial fulfillment of the requirement  
for the degree of Masters of Science in Life Science**

GUIDED BY

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## ***CERTIFICATE***

This is to certify that the thesis report titled “**Production and characterization of Extracellular polymeric substances in Rhizobium with different carbon sources**” submitted by Mr. Sudip Samal to the Department of Life Science, National Institute of Technology, Rourkela in partial fulfillment of the requirement for the degree of Master in Science in Life Science is a bonafide record of work carried out by him under my supervision. The content of this report in full or parts have not been submitted to any other institute or university for the award of any degree or diploma.

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## DECLARATION

I Sudip Samal, M.Sc. Life Science, Department of Life Science, N.I.T., Rourkela hereby declare that my research work incorporated in the dissertation titled “**Production and Characterization of Extracellular polymeric substance of *Rhizobium* with different carbon sources**” is an authentic research work carried at Department of Life science, National Institute Technology, Rourkela under the direct guidance and supervision of Dr. Surajit Das, Asst. Professor, Department of Life science, NIT, Rourkela. The project work is original and no part of this work has been submitted for any other degree or diploma. All the given information is true to best of my knowledge.

Date:

Place: Rourkela

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## **ABBREVIATIONS USED**

Nm = Nanometer

mg = Milligram

ml = Millilitre

μg = Microgram

μl = Microlitre

g = Gram

l = Litre

pH = H<sup>+</sup> ion concentration

cm = Centimeter

mm = Millimeter

min = Minutes

EPS = Extracellular Polymeric Substances

% = Percentage

rpm = Rotations per minute

°C = Degree Centigrade

O.D. = Optical density

PBS = Phosphate buffer saline

BSA = Bovine serum albumin



## ABSTRACT

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Many microorganisms secrete extracellular polymeric substances (EPS) during their life cycle. *Rhizobium* is one such bacteria that secrete EPS at suitable conditions. The production of these EPS is also governed by many growth factors and environmental conditions. One such condition is the carbon (sugar) source that is added in the growth medium. For the *Rhizobium* mannitol is commonly used as the sugar source. But instead sugars like glucose, fructose, sucrose and lactose were also added differently. EPS extracted from the different sugar sources were analyzed. Since the majority of the EPS contains polysaccharides and some amount of proteins, so the samples were tested for the determination of amount of sugars and proteins in various carbon sources. The maximum sugar amount was found in the EPS from mannitol and much protein was derived from the EPS from sucrose. FTIR spectroscopy revealed the presence of functional groups that accounts for its overall structure and functional property. Hence with different carbon sources there will be some variation in the properties of the EPS.

**Keywords :** Extracellular polymeric substances, *Rhizobium*, Carbon sources, FTIR

## **INTRODUCTION**

### **Extracellular polymeric substances**

Microorganisms secrete high molecular weight compounds in the environment known as the extracellular polymeric substances or EPS. These are otherwise known as exopolysaccharides which can remain attached with the bacterial cell's outer surface and released during into its growth medium. The EPS material (Fig.1) majorly comprises of polysaccharides but also contains non-sugar components such as proteins and nucleic acids. These bacterial exopolysaccharides are synthesized in two basic forms of capsular EPS and slime EPS that can be distinguished on the basis of degree of association of the cell surface.

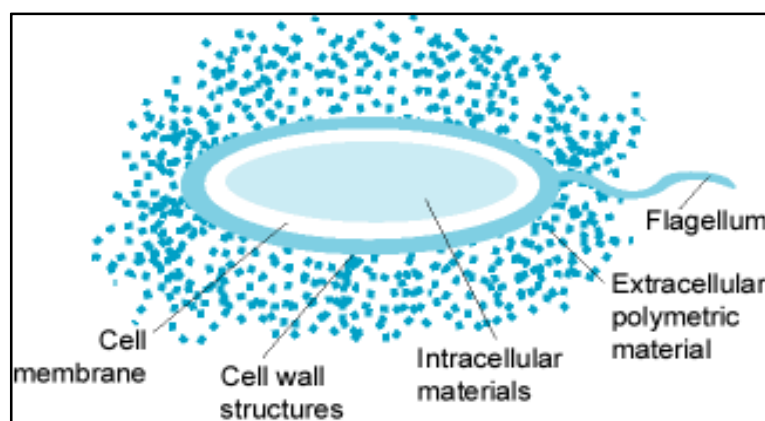


Fig.1. Bacterial cell and EPS

### **General characteristic of EPS**

They are responsible for the architecture and morphology of the matrix in which the cells live. Thus, they can be considered as the microorganism's protective sheet. The EPS has a three dimensional structure, which is gel-like, highly hydrated and sometimes charged matrix. Generally, the proportion of EPS in biofilm varies from 50% to 90 % of the total organic matter (Flemming and Wingender, 2001). EPS originate from microorganisms (excretion and lysis) and wastewater (biosorption). Two types of EPS are identified: capsular and slimy. Capsular substances attached to the cell, whereas the slime is either not bound to the cell, or is free from it. Capsular material plays an important role in the sludge flocculation. The slime is not involved in this process because it is totally free from the cell (Gehr and Henry, 1983). The capsular EPS are held to the cell wall either by linkages between the carboxyl group of EPS and hydroxyl group of lipopolysaccharides (Sutherland, 1977).

Microorganisms produce EPS during their life cycle and the maximal EPS production occurs generally at the end of the growth phase (William and Wimpenny, 1977). In both natural conditions and laboratory microorganisms tend to produce more EPS under nitrogen limiting conditions.

### EPS chemistry

Exopolysaccharides generally consist of monosaccharides and some non-carbohydrate substituents such as acetate, pyruvate, succinate, and phosphate, uronic acid, hexosamines, sulphate esters and generally small amount of lipids and nucleic acids. EPS-producing bacteria are found in a variety of ecological niches. So the physiological role of exopolysaccharides is diverse and may be dependent on the specific natural environment of the organism. The polysaccharide chain might be unbranched or branched with side chains of other compounds attached to the polymeric chain. Generally the polysaccharides are made up of monosaccharides- with hexoses and pentoses forming the majority of EPS. However different monomers contribute to the total polysaccharides varies with the source and such variations in the polysaccharide chain can alter its physicochemical properties. The composition of EPS also largely depends on the extraction method. Table 1. shows the EPS composition and range of component concentration. Table 2. shows the EPS composition of biofilm of *Pseudomonas aeruginosa*.

Table no.1. Compositions of EPS of activated sludge floc and range of component concentration (Flemming and Wingender, 2001)

Component	Content in EPS (%)
Polysaccharides	40-95
Proteins	<1-60
Lipids	<1-40
Nucleic acids	<1-10

Table no.2. Composition of EPS from agar-grown biofilm of *Pseudomonas aeruginosa* (Flemming and Wingender, 2001)

Component	Content in EPS
Total carbohydrate	76.2
Uronic acids	85
Proteins	45.5

The physiochemical properties of EPS are attributed to their diverse and complex chemistry that changes with species diversity, age and growth conditions.

### **Applications of EPS**

A bacterial cell surrounded by EPS is protected from such environmental insults as extreme dry conditions, predation, and antibiotics effects. Bacterial exopolysaccharide likely contribute to such processes as microbial aggregation, surface attachment, biofilm formation, plant-microbe symbiosis, and environmental bioremediation. A major fraction of the primary production is released into the aquatic environments as EPS that contribute to the oceanic dissolved organic matter pool. Recent microscopic and NMR studies have shown that fibrillar polysaccharide formed the bulk of oceanic dissolved organic matter (Santschi et al, 1998).

The distribution of EPS over various size classes, their overall contribution to the total organic carbon pool and their unique properties make them an integral component of marine biogeochemical process including cycling of elements (C, N, S), metals and the marine food web.

Various investigations have been made to study the impact of EPS on fermented milks. It is observed that the EPS produced by LAB modify the microstructure of fermented milks and these changes are then manifested as changes in rheological and other physical properties. In addition to technological benefits certain EPS produced by LAB are also claimed to have beneficial physiological effects on the consumer. It is stated that the increase in viscosity of EPS containing foods may increase the residence time of ingested fermented milk in the gastrointestinal tract and therefore be beneficial to transient colonization by probiotic bacteria. Another example of a suggested health benefit of EPS is the production of short-chain fatty acids (SCFAs) upon degradation in the gut by the colonic microflora.

Exopolysaccharides have a number of industrial applications which relate either directly or somehow to medicine. For example using dextran and its derivatives on both a laboratory and an industrial scale for the purification of compounds of medical benefit, including Pharmaceuticals and enzymes for diagnostic uses. Polysaccharides may also be used for encapsulating drugs for their gradual delivery and they may be used to immobilise enzymes employed for diagnosis or for the chemical modification of pharmaceutical outcomes. This application utilizes the *functional* properties of the polysaccharides, such as rheology or capacity to form gel. Alternatively, the pharmacological and other biological properties of the

polymers may be applied. There are mainly the three types of direct application of the biological properties of exopolysaccharide towards medicine. The exopolysaccharides can be taken as vaccines in preference to whole microbial cells or their cultures. Thus side-effects because of other cell components such as lipopolysaccharides or proteins can be avoided. On the contrary, not all polysaccharides have good immunogenic property, nor the exopolysaccharides necessarily the major factors in the specific disease syndromes caused by the polysaccharide-producing microbial pathogens (Sutherland I.W.,1990)

EPS molecules keep the organism together and are responsible for adhesion to a given surface if they form cell flocculation. One organism can attach to hydrophobic and hydrophilic surfaces by means of different EPS components. Both the adhesion and cohesion property are based on weak physical-chemical interactions and not on covalent bonds. Three major kinds of adhesion force are dispersive forces, electrostatical interactions and hydrogen bonding. The individual of all these interactions is relatively small compared to a covalent C-C bond. However, the total binding energy of weak interaction between EPS molecules multiplied by a large number with binding sites available in the macromolecules add up to bond values exceeding those of covalent C-C bonds. Three types of binding forces are expected to contribute to the overall stability of floc and biofilm. The result is the formation of a three dimensional structure of EPS and the properties may vary dynamically as the microorganism respond to changes in environmental conditions (Flemming and Wingender, 2001). Since EPS are polymer accumulated on the surface of microorganisms, it is a reason that the surface charge is due to the EPS's functional groups, that has either positive or negative charge depending on the nature of groups and pH. At neutral conditions, the functional groups such as carboxylic and phosphate carry negative charge, while those like amino groups carry positive charge.

EPS play important role in in biofilms development in attached-growth process. They act to bridge between cell surface and therefore initiate floc/biofilm formation (Bura et al.,1998).

Among the group of microorganisms that can secrete EPS, the rhizobium bacteria are known to secrete the most. Rhizobium are a type of bacteria that can produce exopolysaccharides in a good quantity which provides its colony a mucoid or slimy appearance in its growth medium.

## Rhizobium

Rhizobium are the soil microorganisms that can survive in the soil or forms a symbiotic association with the host legume by forming root nodules in the roots. Generally they are the Gram negative, motile, non sporulating rod shaped bacteria (Fig.2).



Fig.2. A rhizobium bacterial cell (Frank Dazzo, 1995)

Rhizobia cannot fix nitrogen independently and require a plant host. During the germination of the legume seeds in the soil, the root hairs come in contact with the rhizobia. If rhizobia and the legume are compatible, the rhizobia enters the plant's root hairs by a complex process (Fig.3). Near to the point of entry the plant will develop a root nodule. Infection threads grow to form nodule, infect its cortex and release the rhizobia in those cells, where they undergo differentiation into bacteroids and fix nitrogen from the atmosphere into a form that can be plant usable, ammonium ( $\text{NH}_4^+$ ), using the nitrogenase enzyme. In return, the plant will supply the bacteria with essential nutrients, and sufficient oxygen so as not to interfere with the process of fixation. Leghaemoglobins, the plant proteins are similar to human hemoglobins, helps in providing oxygen for respiration while keeping the free oxygen concentration low enough not to inhibit nitrogenase activity.

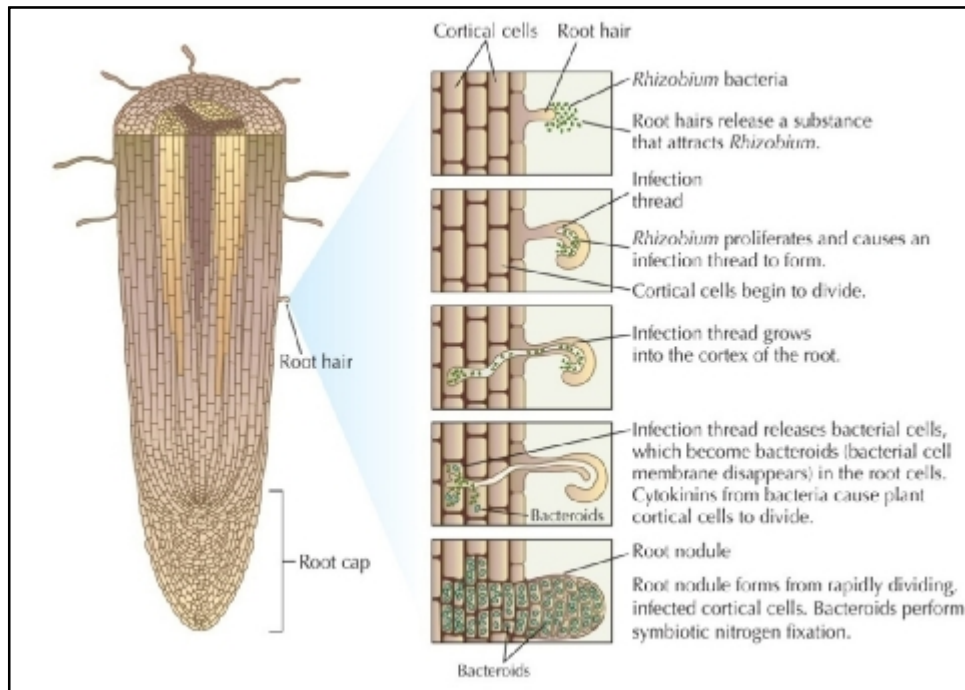


Fig.3. Root nodules formation by rhizobium (Cold spring Harbor Laboratory press, 2007)

The symbiotic relationship implies a signal exchange between both partners that leads to mutual recognition and development of symbiotic tissues. Rhizobia dwell in the soil where they can be able to sense flavonoids secreted by the roots of their host legume plant. Flavonoids trigger the nod factors secretion, which are then recognized by the host plant and can lead to root hair deformation and several cellular reactions, such as ion fluxes. The well-known infection mechanism is called intracellular infection, where the rhizobia gets entry through a deformed root hair in a similar way to endocytosis, forming an intracellular tube which is called as infection thread. A second mechanism is called "crack entry". Here no root hair deformation is seen and the bacteria penetrates in between cells, by cracks produced through lateral root emergence. Later, the bacteria becomes intracellular and an infection thread is formed like in intracellular infections.

*Rhizobium leguminosarum* was the first species to be identified in the year 1889. As far as taxonomy is concerned they fall into two classes of proteobacteria- the alpha- and the beta-proteobacteria (Table no.3). Most belong to the order of Rhizobiales, but several rhizobia occur in specific bacterial orders of the proteobacteria.

Table no.3 Taxonomy of rhizobium and related bacteria.

<b>α - proteobacteria</b>		<b>β - proteobacteria</b>
<b><i>Rhizobiales</i></b>		<b><i>Bulkholderiales</i></b>
<b>Bradirhizobiaceae</b>	<b>Rhizobiaceae</b>	<b>Bulkholderiaceae</b>
<i>B. canariense</i>	<i>R. cellulosilyticum</i>	<i>B. caribensis</i>
<i>B. elkanii</i>	<i>R. daejeonense</i>	<i>B. dolosa</i>
<i>B. japonicum</i>	<i>R. etli</i>	<i>B. mimosarum</i>
<i>B. liaoningense</i>	<i>R. galegae</i>	<i>B. phymatum</i>
<i>B. yuanmingense</i>	<i>R. gallicum</i>	<i>B. tuberum</i>
Brucellaceae	<i>R. giardinii</i>	<i>C. taiwanensis</i>
<i>O. cytisi</i>	<i>R. hainanense</i>	<b>Oxalobacteraceae</b>
<i>O. lupini</i>	<i>R. huautlense</i>	<i>H. lusitanum</i>
<b>Hyphomicrobiaceae</b>	<i>R. indigoferae</i>	
<i>A. caulinodans</i>	<i>R. leguminosarum</i>	
<i>A. doebereineriae</i>	<i>R. loessense</i>	
<i>D. neptuniae</i>	<i>R. lupini</i>	
<b>Methylobacteriaceae</b>	<i>R. lusitanum</i>	
<i>M. nodulans</i>	<i>R. mongolense</i>	
<b>Phyllobacteriaceae</b>	<i>R. miluonense</i>	
<i>M. albiziae</i>	<i>R. sullae</i>	
<i>M. amorphae</i>	<i>R. tropici</i>	
<i>M. chacoense</i>	<i>R. undicola</i>	
<i>M. ciceri</i>	<i>R. yanglingense</i>	
<i>M. huakuui</i>	<i>S. abri</i>	
<i>M. loti</i>	<i>S. adhaerens</i>	
<i>M. mediterraneum</i>	<i>S. americanum</i>	
<i>M. plurifarum</i>	<i>S. arboris</i>	
<i>M. septentrionale</i>	<i>S. fredii</i>	
<i>M. temperatum</i>	<i>S. indiaense</i>	
<i>M. tianshanense</i>	<i>S. kostiense</i>	
<i>P. ifriqiense</i>	<i>S. meliloti</i>	
<i>P. leguminum</i>	<i>S. mexicanus</i>	
<i>P. trifolii</i>	<i>S. morelense</i>	
	<i>S. saheli</i>	
	<i>S. terangae</i>	
	<i>S. xinjiangense</i>	

Rhizobia living in the soil are called saprophytes. Many rhizobium live in soil organic matter without legume partners which are called native rhizobia. When farmers add them as inoculants then they are called introduced rhizobia. The number of native rhizobia in any soil can be very diverse, including many species, and several distinct strains within one species. Numbers can vary from zero to more than a million rhizobia per gram (g) of soil. Several factors affect the number of rhizobia in soil. These include vegetation, cropping history, and environmental and soil conditions. Rhizobia are generally found in large numbers in a region which are close to the plant roots known as the rhizosphere. The population of rhizobia in the



soil is also influenced by rainfall. They generally prefer to live in soils that are moist but not water logged. Soil temperature and acidity are also other important conditions. Soil temperature of around 25° to 30° C and pH of 6 to 6.8 is generally preferred by the bacteria. These are mainly sensitive to low pH (acidic soil).

Rhizobium plays important role in agriculture by inducing nitrogen fixing nodules on the roots of legume plants. This symbiotic association can be beneficial in relieving the requirement for added nitrogenous fertilizer during the growth of leguminous crops. The process of nodule formation and maintenance provides novel opportunity for the study of signal transduction in a plant system.

## **REVIEW OF LITERATURE**

### **EXOPOLYSACCHARIDES**

Studies on bacterial polysaccharide as a viable source of polymeris materials and their use as emulsifiers, stabilizers, binders, coagulants and suspending agents in a variety of industries has been done by Margaritis and Pace (1985). The biochemical pathways leading to exopolysaccharide synthesis are fairly well understood, and can be divided into four distinct steps as described by Sutherland (1982).

- Monosaccharides activation by formation of sugar nucleotides
- Repeating units assembly via addition of sugars to isoprenoid lipid
- Polymerization of repeating units
- Polysaccharide excretion by cytoplasmic membrane and cell wall.

These enzymes have been divided into four groups by Sutherland, broadly corresponding to the four steps of exopolysaccharide synthesis: Group 1, initial substrate metabolism enzymes; Group 2, enzymes whicht synthesize and convert sugar nucleotides; Group 3, transferase enzymes that build monosaccharide units; and Group 4, enzymes which synthesize the exopolysaccharide molecules.

A study has been done by Neihaus *et al.*, (1993) on the composition of the EPS material produced that varies widely from species to species. In addition, EPS synthesis and composition are a function of a variety of environmental factors. Such factors and specific conditions of culture can dramatically impact exopolysaccharide production in terms of EPS yield as well as the size and chemical composition of the polysaccharides being formed.

The factors are like:

- Carbon impact – Studies by Breedveld (1993) have found exopolysaccharide composition to be unchanging with different carbon substrate utilization. while others like Cerning (1994) have found exopolysaccharide composition to be variable upon utilization of different carbon substrates.
- Nitrogen - EPS yields are known to vary based upon the nitrogen substrate utilized as studied by Dalta and Basu (1999).
- Carbon/nitrogen ratio - Various studies by Corpe (1964) have found that EPS production is favored under conditions of nitrogen limitation (high carbon/nitrogen ratios).

- Others nutrients – Bergerson (1960) studied that these nutrients include, but are not limited to, potassium, magnesium, calcium, and phosphate.
- Oxygen availability - Bacterial growth (biomass) tends to increase with higher agitation rates, under conditions where oxygen limitation is not imposed.
- pH – Gorret et al (2001) studied that the optimal pH for exopolysaccharide production may differ from the optimal pH for bacterial growth.
- Temperature – as studied by Farres et al (1997) exopolysaccharide production is often favored by sub-optimal growth temperatures.

The chemical composition of exopolysaccharides was studied in agrobacterium and rhizobium by Zevenhuizen (1971). Hopkins et al (1930) found glucose and glucuronic acid as components of the exopolysaccharides of *Rhizobium meliloti*, *R. leguminosarum* and *R. trifolii*. Schlichterer & Stacey (1945) investigated on the structure of the exopolysaccharide of *R. radicolum* by methylation. Subsequently, composition was studied in relation to taxonomy (Humphrey & Vincent, 1959 ; Graham, 1965) and antigenic properties (Dudman, 1964). Ljunggren & Fihraeus (1959) studied the effect of exopolysaccharides on root-hair entry by rhizobia, while Clapp, Davis & Waugaman (1962) used exopolysaccharides of *Rhizobium* as model compounds to study the importance of polysaccharides in stability of soils.

Effect of different carbon sources on the production of Extracellular Polysaccharide by *Tremella cinnabarina* in submerged culture was studied by Khondkar (2009). The effect of emulsifiers, fatty acids and plant oils were studied focusing on potentiality to support the growth and production of extracellular polysaccharide of *Tremella cinnabarina* in submerged culture. The extracellular polysaccharide (EPS) production and mycelial growth were substantially increased when supplemented with certain fatty acids in the media. Grape-seed oil, Oleic acid, Sorbitan mono-oleate significantly enhanced EPS production in comparison to the control. In lipids, the results showed that the stimulation or inhibition was associated with the lipids type and levels. However, when only lipids were used as sole carbon source, EPS production were poor.

Joshua *et al.*, (2011) studied the variation of exopolysaccharide production in *Rhizobium tritici*. They stated that *R. tropici* chemical composition and yield of EPS differs with

different substrates in a minimal medium in batch culture. Exopolysaccharide quantified from *R. tropici* were grown using arabinose, sucrose, glucose, mannitol, glutamate and fructose. All substrates produced plenty amount of exopolysaccharide substances. Changes in pH and carbon- to-nitrogen (C/N) ratio were also responsible for assorted cell growth and differences exopolysaccharide production. It was found that optimization the C/N ratio has a greater impact upon *R. tropici* production of EPS than upon growth of *R. tropici*. A maximum EPS yield of 4.08 g/L was obtained under optimized conditions, which is greater in comparison with other known rhizobia. Evidence was provided that the chemical composition of *R. tropici* EPS can change with changes to the growth environment. The composition of glucose-extracted EPS contained rhamnose-linked residues which were not in arabinose-extracted EPS.

Malgorzata *et al.*, (2006) studied about rhizobial exopolysachhrides, how they are controlled genetically and their symbiotic functions. This review focused on role of exopolysaccharides in the invasion that leads to formation of indetermined type of nodules on legumes such as clover, peas, vetch, or alfalfa. The significance of EPS synthesis in symbiotic interactions of *Rhizobium leguminosarum* with clover is noticed specially. Accumulation of data suggest that exopolysaccharides may be involved in invasion and development of nodule, bacterial release from infection threads, development of bacteroids, suppression of plant defense response and protection against plant antimicrobial compounds. Exopolysaccharides of rhizobia are species-specific heteropolysaccharide polymers composed of common sugars that are substituted with non-carbohydrate moities. Repeating units' synthesis of exopolysaccharide, modification, polymerization and their export to the cell surface is controlled by clusters of genes that are located on rhizobial megaplasms or chromosome. These genes function was identified by isolation and characterization of several mutants disabled in synthesis of exopolysaccharide. The effect of deficiency of exopolysaccharide on nodule development has been properly studied. Production of exopolysaccharides is influenced by a complex network of environmental factors such as nitrogen, sulphur or phosphate. There is a strong view that production of a variety of symbiotically active polysaccharides may allow rhizobial strains to adapt to changing environmental conditions and interact efficiently with legumes.

Olivier *et al.*, (2008) studied the characteristic and turnover of exopolymeric substances in a hypersaline microbial habitat. The properties and microbial turnover of exopolymeric

substances (EPS) were measured in a hypersaline nonlithifying microbial mat (Eleuthera, Bahamas) to investigate their potential role in calcium carbonate ( $\text{CaCO}_3$ ) precipitation. Deep profiles in EPS abundance and enzyme activities indicated that in the upper 15–20 mm 80% EPS were turned over. Oxic as well as anoxic homogenates amended with low-molecular-weight (LMW) organic carbon, sugar units and different varieties of EPS showed rapid consumption of all substrates. When the consumption of EPS was compared with that of other substrates, just marginal longer lag times along with lower rates were found. EPS (5–8%) were readily consumed during the conversion of labile to EPS refractory. This matched with a decrease in glucosidase activity and a decrease in the number of acidic functional groups of EPS. About half of the calcium bound to the EPS remained after 10 dialyses steps. This tightly attached calcium was readily available for precipitation of  $\text{CaCO}_3$ . A conceptual model presented LMW organic carbon complexed with the tightly bound calcium that was released upon enzyme activity. This increased alkalinity and created binding sites for carbonate and allows  $\text{CaCO}_3$  precipitation. Therefore, this model will explain bondind between EPS and  $\text{CaCO}_3$  precipitation, and explains the critical role of aerobic and anaerobic microorganisms in early diagenesis and lithification processes.

Gholami et al., (2009) studied about the effect of plant growth promoting rhizobia on germination, seedling growth and yield of maize. The effect of plant growth-promoting rhizobacteria (PGPR) on germination of seeds, growth of seedling and field grown maize yield were evaluated in 3 experiments. In these experiments 6 bacterial strains including *P.putida* strain R-168, *P.fluorescens* DSM 50090, *A.lipoferum* DSM 1691, *P.putida* DSM291, *P.fluorescens* strain R-93 and *A.brasilense* DSM 1690 were used. Results of first study showed that inoculation of seed significantly enhanced seed germination and seedling vigour. In 2nd experiment, dry weight of leaf and shoot and leaf surface area were increased by bacterial inoculation significantly in both non-sterile and sterile soil. The results stated that inoculating with bacterial treatments had a more stimulating effect on growth and development of plants in nonsterile than sterile soil. In the third experiment, maize seeds inoculation with all bacterial strains significantly increased height of the plant, seed weight of 100, no. of seed per ear and leaf area. The results also showed significant increase in ear and shoot dry weight of maize.

Zehra et al., (2008) studied the influences of different carbon sources on exopolysaccharide production. Exopolysaccharides (EPSs) production was studied by *Lactobacillus delbrueckii*

subsp. *bulgaricus* (B3, G12) and *Streptococcus thermophilus* (W22) in the medium containing various carbon sources (glucose, sucrose, fructose, or lactose). For all strains, glucose was the most efficient one. Also, the influence of different concentrations of glucose (5,10,15,20,25,30 g/L) on EPS production and growth was done. The results showed that EPS production and growth were stimulated by the high glucose concentration (30 g/L).

Wilbert et al., (1998) studied the role of exopolysaccharide in the infection thread during nodulation. Mutants of *Rhizobium leguminosarum* bv *Viciae* bacteria which are affected in the synthesis of exopolysaccharides (EPS) are unable to effectively nodulate their host plants. By studying defined mutants, it was revealed that *R. leguminosarum* bv *viciae* strains required EPS for formation of infection threads in *Vicia sativa* (vetch) as well as for efficient induction of tight curling of root hair. Coinoculation experiments result with the EPS-deficient mutant of *R. leguminosarum* bv *viciae* in combination with heterologous EPS-producing strains indicated that vetch has certain structural requirements for rhizobial EPS to function in symbiotic relationship. It was hypothesized that EPS accelerates root hair curling and infection to such an extent that rhizobial root penetration precedes a plant defense response.

Arora et al., (2010) studied the effect of cyanobacterial exopolysaccharides on salt stress alleviation and seed germination. Effect of exopolysaccharides (EPS) produced by a consortium of cyanobacteria on germination of 3 cereals wheat, maize and rice were studied at different concentration of salt. EPS production was found to be stimulated by salts, which in turn had a significant Na<sup>+</sup> removal capability from aqueous solution. germination of seeds, vigor index and efficiency of mobilization in all the three crops improved when cyanobacterial EPS was applied. While germination had significant improvement by 13 to 30%, mobilization efficiency increased 1.03 to 1.1 times marginally and increase in vigor index by 1.15 to 2.4 times in response by these crops to EPS under non-saline conditions. Salinity showed inhibition effect germination of seed in all species showing 18 to 54% reduction. However, when EPS was there, the salt induced inhibition minimized to 13 to 18%. Inhibitory effect of salt on concentration of chlorophyll, vigor index and efficiency of mobilization of seedlings was reduced in these crops in presence of EPS, marking the latter's role in alleviation of salt stress.

Rihua et al., (2010) conducted identification, screening and statistic optimization of a exopolysaccharide producing *Lactobacillus paracasei* HCT. The exopolysaccharides (EPS) producing ability was determined by phenol-sulphuric acid method and the *Lactobacillus* HCT was the highest EPS-producing strain, that was isolated from Bama centenarian feces in Guangxi of China. *Lactobacillus paracasei* HCT was identified with carbohydrate assimilation profiling and 16S rRNA gene sequence. Preliminary one factor tests were applied to get the favorable conditions for EPS production and microorganism in a chemically defined medium and observed that carbon nitrogen ratio (C/N ratio), cultivation time and temperature had the major influences. Box–Behnken design of experiment and response surface methodology (RSM) was adopted for further studying the interactive effects of these 3 variables on EPS yield and growth of cells. The optimal culture conditions for EPS production were: C/N ratio of 9.090, cultivation time of 60.67 h and temperature of 29.2°C. In such conditions, the maximum EPS yield was 39.0736 mg/ml that was 4 times more than the original production. But the optimal requirement for cell growth were: C/N ratio 8.643, 58.75 h and 31.9°C.

Xu et al., (2007) standardised procedures for extracting, purifying and characterizing of exopolymeric materials from 2 bacteria ( *Segittula stellata* and *Pseudomonas fluorescens* Biovar II) in relevance to actinide binding in aquatic environments. The extracellular EPS of marine bacterium *Sagittula stellata* and soil bacterium *Pseudomonas fluorescens* Biovar II, were extracted by 6 methods, efficacies of which was compared based on the EPS production, composition as well as disturbance of cell. Purification procedures on these EPS were also improved, which turned to be more cost-effective and had less broth interference, compared to previously used methods. Size exclusion chromatography (SEC) evolved as a useful tool, to provide the EPS “fingerprints” extracted by different methods or after each step of purification. Studies on EPS production and its composition at different growth stages provided abundant information and formed a basis for in-depth studies. Results from SEC showed that the bacterial EPS had a constant molecular weight distribution all through the life but with various polymers in various proportions. Three fractions were isolated successfully by a combination of SEC and anion exchange chromatography for “non-attached” EPS produced by *Pseudomonas fluroescens* Biovar II. Protein was found to be a major component of EPS in their native states, that when mixed with the broth material couldn’t be recognized previously. The EPS harvested at the optimal time of the bacterial life was purified according to the improved method and was more enriched in polysaccharides, with small

amounts of proteins, giving the molecules amphiphilic nature. Additionally, simultaneous determination of neutral sugars and uronic acids by GC-EI-MS provided more information on the exopolysaccharides' monosaccharide composition. Spectra of Isoelectric focusing (IEF) of the bacterial EPS spiked with Pu/Th, and Pu-enriched Rocky Flats Environmental Technology Site (RFETS) soil organic colloid spiked with Th showed similar activity distributions of both actinides along the gradient of pH, including activities of both actinides focus on the low pH area. Pu-enriched IEF extract characterization from RFETS soil by spectrophotometric methods and ATR-FTIR stated the co-existence of lipids, proteins and polysaccharides, in dissimilarity to EPS of bacteria, that showed a simpler composition. This suggests that Th/Pu binding to organic macromolecules is more determined by the availability of binding functional groups rather than the exact specific compounds.

Edward et al., (2011) worked on the Detection of Exopolysaccharides/Bioemulsifier Producing Bacterial Isolates from Petroleum Contaminated Soil. Petroleum spill is a major concern for the environment and affects the native microorganism's survival by generation of extreme stress. This study involves isolation of pigmented bacteria from contaminated soil and characterized by 16S rRNA & tested biochemically. Screening of bacteria was done for the secretion of exopolysaccharides (EPS) and bioemulsifiers. Partially purified EPS were taken for chemical analysis and Fourier transform infrared (FT-IR) spectroscopy. Chemical analysis showed the presence of proteins and sugars in EPS. FTIR analysis showed that the EPS is a heteropolymeric polysaccharide with carboxylic, hydroxyl and alkyl functional groups. The bacterial isolate had ability to emanate bioemulsifier that was analyzed by drop collapse, oil displacement test and emulsification index. All the tests have considerably showed that the isolate can secrete bioemulsifier. The results of the study contribute significantly towards the applications of EPS in the bioremediation of hydrocarbon impacted soil.

Tsuneda et al., (2003) studied that Extracellular polymeric substances can help in bacterial adhesion in any solid surface. Extracellular polymeric substances (EPS) influencing on bacterial cell adhesion onto solid surfaces was researched using 27 heterotrophic bacterial strains from a wastewater treatment reactor. Cell adhesion on glass beads was done by the packed-bed method and the results were analyzed in terms of quantity of each EPS component obtained and characteristics of cell surface such as zeta potential and hydrophobicity. Protein and polysaccharides formed 75-89% of the EPS composition,



indicating major EPS components. Among the polysaccharides, hexose, hexosamine and ketose amounts were relatively high in EPS-rich strains. For strains poor in EPS, the cell adhesion efficiency onto glass beads increased as the values of zeta potential decreased, viewing that electrostatic interaction can suppress cell adhesion efficiency. While the amounts of hexose and pentose showed good correlations with cell adhesiveness in EPS-rich strains, stating that polymeric interaction by EPS covering on the cell surface provided cell adhesion. It was concluded that, if the amount of EPS is relatively small, cell adhesion onto solid surfaces can be inhibited by electrostatic interaction, and when relatively large, cell adhesion is increased by polymeric interaction.

Joo et al., (2004) studied the optimization of submerged culture conditions for exopolysaccharides production in *Sarcodon aspratus* (Berk) S.lto TG-3. The effect of medium components (carbon, nitrogen, and mineral sources) and environmental factors (initial pH and temperature) for mycelial growth and exopolysaccharide (EPS) production in *Sarcodon aspratus* (Berk) S.lto TG-3 was investigated. The optimal temperature (25 °C) and initial pH (5.0) for the EPS production in *S. aspratus* shake flask cultures were determined using the two-dimensional contour plot. The most suitable sources of carbon, nitrogen, and mineral sources for producing EPS were glucose, CaCl<sub>2</sub>, yeast extract and KH<sub>2</sub>PO<sub>4</sub>, respectively. Remarkably, the EPS production was significantly enhanced by calcium ion supplementation. Subsequently, the optimum concentration of glucose (30 g l<sup>-1</sup>), yeast extract (15 g l<sup>-1</sup>), CaCl<sub>2</sub> (1.1 g l<sup>-1</sup>), and KH<sub>2</sub>PO<sub>4</sub> (1.2 g l<sup>-1</sup>) were determined by orthogonal matrix method. The nutritional requirement effects upon mycelial growth of *S. aspratus* were in continuous sequence of glucose > KH<sub>2</sub>PO<sub>4</sub> > yeast extract > CaCl<sub>2</sub>, and those on EPS production were in the order of glucose > yeast extract > CaCl<sub>2</sub> > KH<sub>2</sub>PO<sub>4</sub>. Under the optimal conditions for culture, the EPS concentration was maximum in a 5-l stirred-tank reactor was 2.68 g/l, 4 days after fermentation, which was 6- fold higher than that of basal medium. Contour plot and orthogonal matrix of two-dimensional method allowed to find the relationship between environmental factors and nutritional requirement by making out optimal operating conditions for maximum EPS production in *S. aspratus*. The statistical experiments applied for this work can be useful strategies for optimization of submerged culture processes for other mushrooms also.

## **RHIZOBIUM**

Graham (1969) had reported about a new medium developed for selectively isolating strains of Rhizobium from soil. Fast-growing strains of rhizobia incubated on YMA for 3 to 5 days at 28 C produced smooth, white, glistening colonies measuring 1 to 3 mm in diameter. Slow-growing rhizobial colonies had less than 1 mm diameter, but were also white, glistening, and raised. Albizo and Surgalla used a selective medium that contained antiserum to isolate and identify fraction 1-positive *Pasteurella pestis*. When specific antirhizobial serum is incorporated into the YMAA medium, it was used to identify and quantitate rhizobial strains from soil. This would be useful in studying strain persistence and competition. If the strains used in such studies are carefully selected, the method efficient than existing ways to assess soil rhizobia. The medium also has usefulness for isolating rhizobiophage and suitable host rhizobia from soil.

One isolation method was followed by Pham Quang Hung and K. Annapurna (2004) in which they isolated and characterized endophytic bacteria in soyabean (*Glycine sp.*). Plant-associated bacteria which survive inside plant tissues without causing any harm to plants are known as endophytic bacteria. This investigation was done to analyze the phenotypic and genotypic diversity in bacterial endophytes of two species of soybean viz. *Glycine max* and *G. soja*. 65 number of bacterial endophytes were isolated from three tissues: stem, root and nodule. These isolates were screened for Gram reaction, secretion of hydrolytic enzymes like pectinase and cellulase, production of fluorescent pigment, and motility, resistance to streptomycin, formation of capsule and IAA production. Genotypic variation was studied by PCR-based 16S rDNA-RFLP. Preliminary characterization of 65 endophytes revealed that approximately same percentages of gram positive and gram negative bacteria were present. Approximately 80% of them were motile, 33% and 70% secreted pectinase and cellulase, respectively and in 17% no IAA was produced in vitro. 65 isolates were phenotypically found to show less closeness among them in the characters studied. Molecular characterization of selected bacteria was done by PCR amplification of 16S rDNA gene, and along with the restriction analysis.

Abdelal et al., (2003) studied the molecular diversity and identification of free living strains of rhizobia. Soils from Delta Nile Valley and the Al Ismailyah region of Egypt were examined to know the dominant, independent of trap-host, species of Rhizobium by using direct soil isolation technique. Isolation of 34 Egyptian Free living Rhizobial Isolates

(EFLRI) was done from these soils without the use of a trap host and species status was known by using partial sequencing of *16S rDNA*. *16S rDNA* sequences and phylogenetic analyses showed that 38.2% of strains were identified as *Sinorhizobium meliloti*, 29.4% were related to *Sinorhizobium medicae*, 23.5% as *Agrobacterium tumefaciens* and 8.8% of them taxonomically similar to *Rhizobium etli*. Analysis by Amplified ribosomal *16S rDNA* revealed that strains of *Sinorhizobium medicae*, *Agrobacterium tumefaciens* and *Rhizobium etli* each related to two different genotypes while strains of *Sinorhizobium meliloti* related to one genotype which was identical to the standard strain *S. meliloti* 2011. The EFLRI strains remainder, with the exception of the three *Rhizobium etli* strains, failed to produce amplified fragment with *nodC* primers showing that free *Rhizobium* strains existing in the soil without their legume hosts often lack the symbiotic genes. ARDRA results and *16S rDNA atpD* and *glnII* genes sequence analysis showed that the most dominant species in these soils was *S. meliloti* and this species may be present in the rhizosphere of wheat plants.

Akiko *et al.*, (2006) studied on Bioremediation of cadmium contaminated soil using symbiosis between leguminous plant and recombinant rhizobia with the MTL4 and the PCS genes. Cadmium contamination in rice grains is a major issues in Asian countries. They had developed a novel bio-remediationsystem based on leguminous plant and genetically engineered rhizobia symbiosis. They designed two types of recombinant rhizobia, having two genes, MTL4 and AtPCS. The MTL4 and AtPCS genes put to *Mesorhizobium huakuii* subsp. *rengei* B3, that can infect and form nodules on Chinese milk vetch, *Astragalus sinicus*. The two genes were combined to the *nolB* or *nifH* promoter, that generated nodule specific gene expression in strain B3. The two recombinant strains, B3(pMPnolBMTL4nifHPCS) and B3 and nifHMTL4(pMPnifHPCS), revealed 25 and 12-fold increase in Cd concentration, in free-living cells, respectively. When the symbiotic relationship was established with the strains with *A. sinicus*, the symbionts increased accumulation of Cd in nodules by two-fold in hydroponic culture. Both MTL4 and AtPCS genes expression showed additive effect on cadmium accumulation in nodules. They had also used these recombinant bacteria to rice paddy soil polluted with Cd (1 mg kg<sup>-1</sup> dry weight soil). The Cd accumulation increased not only in nodules but also in *A. sinicus* roots infected by the recombinant rhizobia. The Cd accumulation in the plant roots infected by B3(pMPnolBMTL4nifHPCS) gained three-fold than that by the wild-type B3. After two months of cultivation of the symbiont, Cd was removed from the paddy soil about 9%. Thus, the symbiosis was useful in phytoremediation for heavy metals.

Zevenhuizen (1971) worked on chemical composition of exopolysaccharide in both rhizobium and agrobacterium. Hopkins et al (1930) found glucose and glucuronic acid as constituents of the exopolysaccharides of *Rhizobium meliloti*, *R. leguminosarum* and *R. trifolii*. Schlichter & Stacey (1945) investigated the exopolysaccharide structure of *R. radicicolum* by methylation. Subsequent composition was studied in relation to taxonomy by Humphrey & Vincent (1959) and Graham (1965) and to antigenic properties by Dudman, 1964. Ljunggren & Fihraeus (1959) studied the exopolysaccharides effect on root-hair invasion by rhizobia, while Clapp *et al.*, (1962) used *Rhizobium* exopolysaccharides as model for studying the importance of polysaccharides in soils' crumb stability.

Caviedes *et al.*, (1982) studied that many of the rhizobia are known to produce good amount of EPS material both in the rhizosphere as well as in pure culture. Kaci et al studied that exopolysaccharide in rhizobia serve as soil aggregate. It protect the bacteria during environmental stress as studied by Becker and Puhler (1998). Leigh et al (1985) studied that exopolysaccharide is important as a structural component of root nodules. It helps to increase the competitiveness in the nodulation process as shown by Morin (1998).

## **OBJECTIVE**

The primary aim of this project work was to study about the variations observed in the production of the extracellular polymeric substances (EPS) that were extracted from different carbon sources taking *Rhizobium* as the bacterial sample. Different sugars were used as carbon source and the bacterium was isolated from root nodules of legume plant. Different sugars were added in the medium and EPS were extracted to be analyzed biochemically. To fulfill the objectives the research work was proceeded by the following steps:

- § Collection of root nodules from legume plant
- § Isolation of *Rhizobium* bacteria
- § Extraction of the Extracellular polymeric substances (EPS) from bacteria with different carbon sources.
- § Characterization of the EPS
  - Biochemical characterization- for carbohydrates and proteins
  - FTIR analysis

## **MATERIALS AND METHOD**

### **1. Sample collection:**

- Legume plants of *Vigna radiata* were planted.
- Root nodules were seen to occur in plants after 3-4 weeks.
- Those were uprooted carefully and collected in a sterile aluminum foil and brought to the laboratory.

### **2. Sterilizing the sample:**

- Those few plants were washed under tap water to remove soil and separated into stems, roots and nodules.
- Stems and roots were cut into sections 2-3 cm long.
- The tissue was put in beaker, soaked in distilled water and drained.
- The root nodules were carefully removed along with some parts of roots.
- It was rinsed in 70% ethanol for 30 seconds and then sterilized with 0.1% HgCl<sub>2</sub> for 3 minutes.
- The tissue was then washed ten times with sterile water (Gagne et al., 1987).

### **3. Bacterial isolation:**

#### **Materials required:-**

Beakers

Homogenizers (mortar and pestle)

Conical flask

Test tubes

Vortex

#### **Method:-**

- Surface-disinfected tissue was aseptically macerated with homogenizers.
- Macerated tissue was diluted into 10<sup>-1</sup> dilution by adding 9 volumes of sterile distilled water.
- Serial dilution was made up to 10<sup>-4</sup> dilution by taking 1 ml of well-shaken suspension and adding into 9 ml water blank tubes.
- 100 µl from 10<sup>-4</sup> dilutions were spread plated on the selective media.



Fig.4. seeds of *Vigna radiata* (moong dal)



Fig.5. root nodules in the roots of *Vigna radiata*



Fig.6. serial dilution of the sample

**Preparation of media-** To isolate soil bacterium mainly rhizobium Yeast Extract Mannitol Agar was prepared. Its composition is as follows-

Table no.4 Standard composition of media.

composition	Amount (g/l)
Yeast extract powder	1
Mannitol	10
Magnesium sulfate	0.2
Dipotassium hydrogen phosphate	0.5
Sodium chloride	0.1
Calcium carbonate	1
Agar	15

Accordingly the media was weighed and prepared for pouring into plates (300 ml). hence the ingredients will be as-

Table no.5 Media composition in the experiment

Yeast extract powder	0.3 g
Mannitol	3 g
Magnesium sulfate	0.06 g
Dipotassium hydrogen phosphate	0.15 g
Sodium chloride	0.03 g
Calcium chloride	0.3 g
Agar	4.5 g

- The media was prepared in a media bottle and autoclaved.
- Once autoclaved these were poured in the sterile petriplates.
- After the media got solidified it was kept for incubation for overnight to watch out for contamination.



### **Spreading-**

- Once there was no contamination, the plates were taken to the laminar air flow chamber.
- 100 µl was taken from the  $10^{-3}$  and  $10^{-4}$  dilution and was pipetted out in the petriplates.
- With the help of sterile spreader or L-shaped rods it was thoroughly spreaded.
- Once the spreading was done the plates were kept inside the incubator for 2-3 days incubation to see for the growth of bacterial colony.

### **Confirmation of desired bacteria**

The rhizobium colony was identified on the basis of the colony morphology and streaked on a separate plate.

#### Colony morphology –

- Shape- round
- Colour- white
- Appearance- slimy or mucoid

#### GRAM STAINING :

- a clean glass slide was taken and wiped with alcohol.
- From the streaked colony a loop was touched with the culture and rubbed in the slide
- A very thin smear was prepared and slightly heat fixed.
- It was flooded with crystal violet and kept for 1 minute.
- It was gently washed and flooded with Gram's iodine
- After keeping for 1 minute it was again washed.
- Decolorizer (95% ethyl alcohol) was added until the purple dye no longer flows from the smear.
- It was again was gently with water.
- Counterstain Safranin was added and kept for 30 seconds.
- Finally it was washed with water and viewed under microscope.

- Rhizobium was known by the shape and the type of stain it took.

#### GROWTH ON CONGO RED AGAR :

Table 4. COMPOSITION OF CONGO RED AGAR

Composition	g/l
Yeast extract	1
Mannitol	10
Dipotassium phosphate	0.5
Magnesium sulphate	0.2
Sodium chloride	0.1
Congo red	0.025
Agar	20
pH	6.8±0.2

The bacteria was streaked on the YEM Congo red agar plate and kept for incubation to observe the bacterial growth.

#### GROWTH ON YEM AGAR + Bromothymol blue :

On the YEM agar, 0.25 mg of Bromothymol blue was added. Then bacteria was streaked on it.

Once the confirmatory tests were done, slants of YEM agar were prepared in the test tubes and the rhizobium culture was inoculated in it so as to preserve the culture for further analysis.

### **4. EPS extraction :**

#### **Preparation of YEM broth :**

YEM broth (100 ml) was prepared without the addition of any sugar compound and agar.

Yeast extract	0.1
Di-potassium hydrogen phosphate	0.05

Calcium chloride	0.1
Sodium chloride	0.01
Magnesium sulphate	0.02

These were transferred in five Falcon tubes and autoclaved.

### **Preparation of sugar stock solution**

- Five autoclaved test tubes were taken.
- 20% sugar stock solution was to be prepared.
- In the five test tubes 10 ml of distilled water was taken.
- 2 mg of five different sugars i.e. Glucose, Fructose, Sucrose, Lactose and Mannitol were dissolved.
- These were thoroughly mixed.
- Again five clean test tubes were taken.
- With the help of syringe filter sugars from respective stock were transferred into the clean tubes.
- Around 3 to 5 ml were collected through syringe filters.

### **Adding sugars to the YEM broth**

- The five falcon tubes in which the broth was transferred were taken to the laminar.
- The five tubes containing the filtered sugars solution were also kept at the laminar.
- 200  $\mu$ l (1%) of sugar solution was added to the broth with the help of micropipette.
- These were kept in the freeze for further use.

### **Bacterial inoculation**

- The five broth tubes with five different sugars were taken in the laminar chamber.
- The bacterial culture was taken inside the chamber.
- The inoculating loop was heated in the flame till red hot.
- A loopfull of culture was inoculated in the falcon tubes containing broth.
- In all the falcon tubes, containing different sugars, loopful of culture was inoculated.
- It was kept for three days incubation, at room temperature with constant shaking.
- So the falcon tubes were kept in the shaker for three days.

- After this was to be used for the extraction of EPS.
- After three days the falcon tubes were centrifuged at 7500 rpm at 4°C for 10 minutes.
- Once the cells get separated the supernatant was taken in another falcon tubes.

### **EPS precipitation by ethanol**

- In those tubes containing the supernatant, about twice the volume of chilled ethanol was added to get the EPS in precipitated form.
- So after the addition of chilled ethanol the falcon tubes were kept overnight.
- After an overnight incubation, the tubes were centrifuged at 10,000 rpm for 10 minutes at 4 C.
- The supernatant was discarded and the pellet was kept for drying.
- The dry pellet was the extracted EPS.
- Once dried it was properly scraped and kept in the micro centrifuge tubes for various analysis.

### **5. HI-Carbo Kit test**

The test was done to test the sugar utilization of the bacterial sample in three parts- A,B and C

### **6. Analysis of EPS**

#### **CARBOHYDRATE ESTIMATION (BY PHENOL SULHURIC METHOD) :**

- Firstly a standard curve of glucose was drawn by taking the sugar in various concentration.

#### **Chemicals required:**

- Glucose stock solution (200 µg/ml)
- 5% phenol
- Sulphuric acid
- Distilled water

**Materials required :**

- test tubes
- test tube stand
- water bath
- spectrophotometer

**Method :**

- First a stock of glucose was prepared with a concentration of 200 µg in 1 ml of solution.
- Now the test samples were prepared by changing the concentration of glucose such as 20µg, 80µg, 120µg, 160µg and 200µg.
- Then 400 µl of different glucose concentration were taken in test tubes in triplicates.
- To this 400 µl of 5% phenol was added.
- Then carefully 2 ml of con.H<sub>2</sub>SO<sub>4</sub> was added.
- These solution was kept in water bath at room temperature for 15 -20 minutes.
- Then the O.D. was taken at 490 nm.
- And from the data obtained, a standard curve of glucose was prepared.

**Carbohydrate estimation using different carbon sources.****Chemicals required :**

- EPS extract with different sugars- glucose, fructose, sucrose, mannitol, lactose
- 5% Phenol
- Con.H<sub>2</sub>SO<sub>4</sub>
- Distilled water

**Materials required :**

- test tubes
- test tube stand
- water bath
- spectrophotometer

**Method :**

- The EPS extract from different sugars were taken in the micro centrifuge tubes and dissolved in 1 ml of autoclaved distilled water.
- Ten clean test tubes were taken.
- Each solution was taken in duplicates, so for each sugar two test tubes were taken.
- From the solution 400  $\mu$ l was taken in test tube.
- To it 400  $\mu$ l of 5% phenol was added.
- And finally Con.  $\text{H}_2\text{SO}_4$  was added
- This were vortexed properly.
- Then the whole solution mixtures were kept in water bath at room temperature.
- And finally the O.D. was taken.
- The amount of carbohydrate could be estimated comparing the values obtained after O.D. measurement by the standard curve of sugar already done.

**PROTEIN ESTIMATION:**

Prior to protein estimation a standard curve of BSA was drawn by Bradford method.

**Chemicals required:**

- BSA
- PBS (1X)
- Bradford reagent
- Distilled water

**Materials required:**

- Test tubes
- Test tube stand
- Micropipette
- Autoclaved tips
- Spectrophotometer

**Method:**

- First of all, the PBS buffer and Bradford reagent was prepared.
- BSA stock was prepared.

- Various concentration of BSA was prepared such as 20 µg/ml, 40 µg/ml, 60µg/ml, 80µg/ml and 100 µg/ml were taken in triplicates in fifteen test tubes.
- 5 ml of Bradford reagent was added to each.
- Then it was kept at room temperature in water bath for 10 minutes.
- Finally O.D. was taken at 590 nm.
- Using the data a standard curve of BSA was plotted.
- By this curve we can estimate the protein concentration present in the five different sugars.

### **Protein estimation of EPS from different sugars :**

#### **Chemicals required:**

- Bradford reagent
- PBS buffer
- EPS extract from different sugars
- Distilled water

#### **Materials required:**

- Ten test tubes
- Test tube stand
- Micropipette
- Water bath
- Spectrophotometer

#### **Method:**

- The EPS extract from different sugars were dissolved in 1 ml of PBS.
- In ten clean test tubes the stock protein solution (EPS + PBS) was added.
- In it corresponding PBS solution was added.
- In each of the test tube, 5 ml of Bradford reagent was reagent was added.
- These were kept in water bath at room temperature for 10 minutes.
- Then finally O.D. was taken at 595 nm.
- From the graph amount of protein was estimated.

## **FTIR ANALYSIS**

- The samples were taken and mixed with Potassium bromide with the help of a mortar and pestle.
- It was then subjected to a pelletizer to form a pellet.
- The pellet was then kept inside the FTIR for analysis.
- A graph was obtained showing the peaks for different functional groups



Fig. 7 FTIR Instrument



## RESULTS

### 1. Plates after spreading with $10^{-4}$ dilution

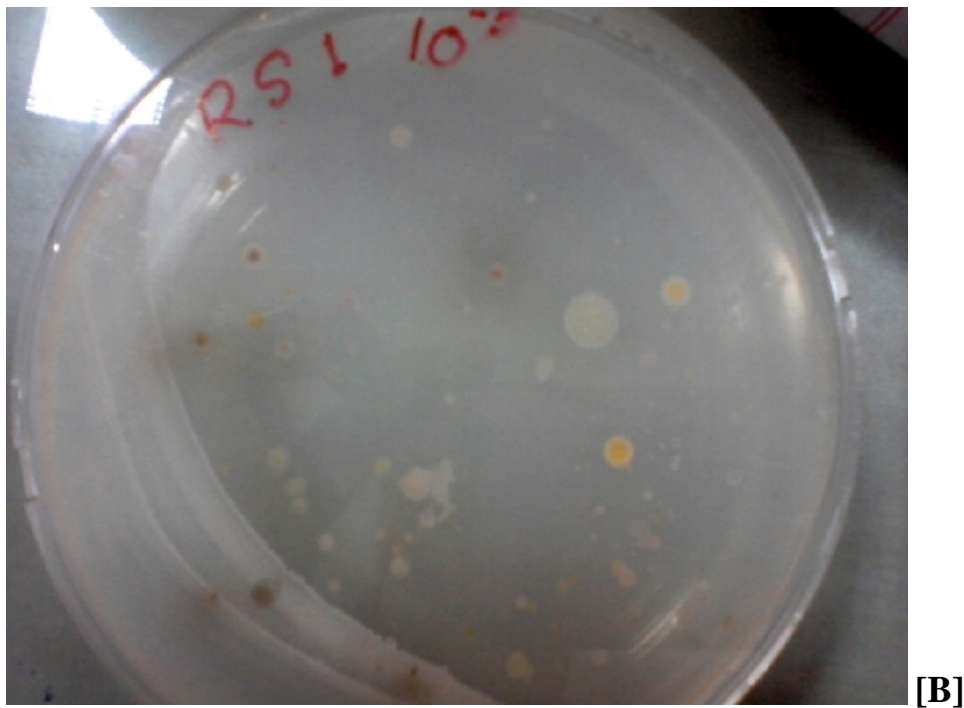
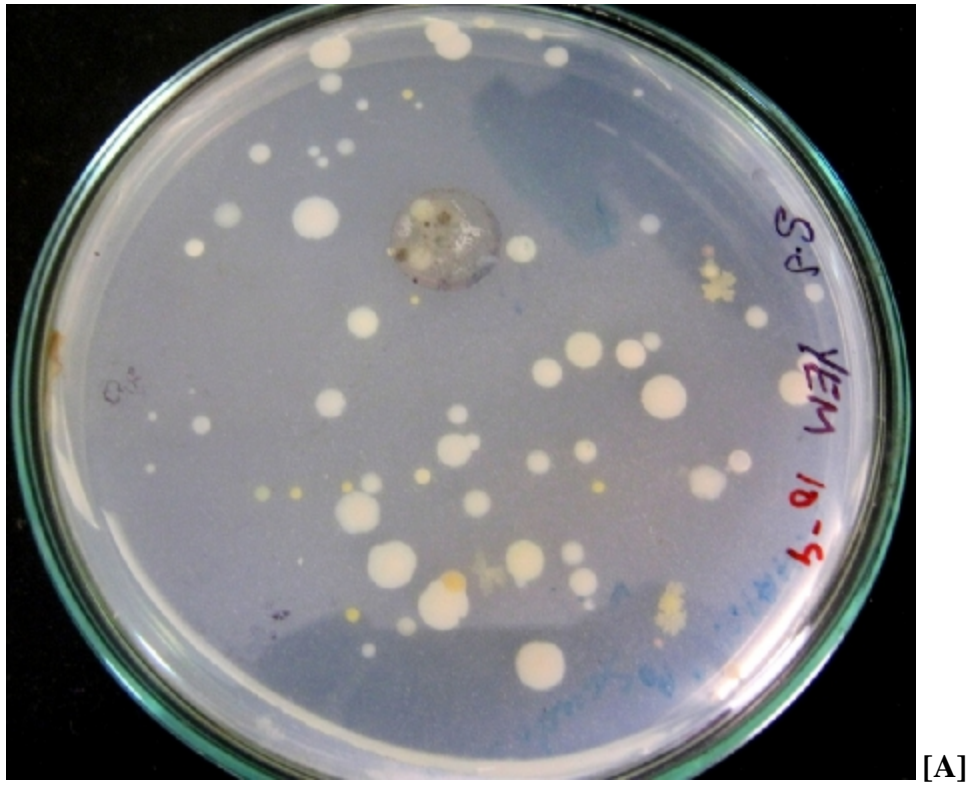


Fig.8 [A] plates with  $10^{-4}$  dilution [B] plates with  $10^{-3}$  dilution

## 2. Growth in Yeast extract mannitol agar

The colony was found to be whitish and slimy in appearance when streaked in YEM agar plate.



[A]



[B]

Fig.9 [A] rhizobium streaked in YEM agar plate [B] Its duplicate

### 3. Growth on YEM agar plus Congo red

When the bacteria is streaked in YEM plus Congo red agar, the bacterial colony develops as a pinkish white color.



Fig.10 bacteria streaked in YEM agar + Congo red

### 4. Gram staining

On staining and viewing under microscope the rhizobium colony appears to be bacillus in shape and pink in color signifying Gram negative.

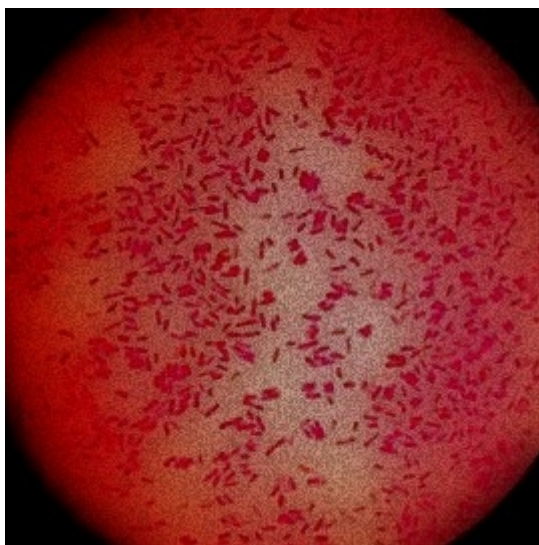


Fig.11 Stained bacteria as seen under microscope

## 5. HI-CARBO KIT Test

Table no. showing the tests and their result

Test	Result
Lactose	+
Xylose	+ -
Maltose	+
Fructose	+
Dextrose	+ -
Galactose	+
Raffinose	+ -
Trehalose	+
Melibiose	+
Sucrose	+
L- arabinose	+
Mannose	+
Insulin	-
Sodium gluconate	-
Glycerol	+
Salicin	-
Dulcitol	-
Inositol	-
Sorbitol	-
Mannitol	-
Adonitol	+
Arabitol	-
Erythritol	-
$\alpha$ – Methyl-D-glucoside	-
Rhamnose	-
Cellobiose	-
Melezitose	-
$\alpha$ –Methyl-D-mannoside	-
Xylitol	-

<b>ONPG</b>	-
<b>Esculin hydrolysis</b>	+
<b>D-arabinose</b>	-
<b>Citrate utilization</b>	-
<b>Malonate utilization</b>	-
<b>Sorbose</b>	-



Fig.12 Hi-carbo kit test

## 6. ANALYSIS OF EPS

### A. Carbohydrate estimation:

#### A standard curve for carbohydrate

Table no.8 Observation table with glucose as standard sugar

Concentration (µg/ml)	working stock volume(µl)	d.w.(µl)	phenol(µl)	sulfuric acid(ml)	absorbance at 490 nm			mean
40	200	800	400	2	0.309	0.317	0.34	0.322
80	400	600	400	2	0.513	0.549	0.446	0.503
120	600	400	400	2	0.791	0.837	0.7	0.776
160	800	200	400	2	0.994	0.993	1.014	1
200	1000	0	400	2	1.791	1.313	1.279	1.461

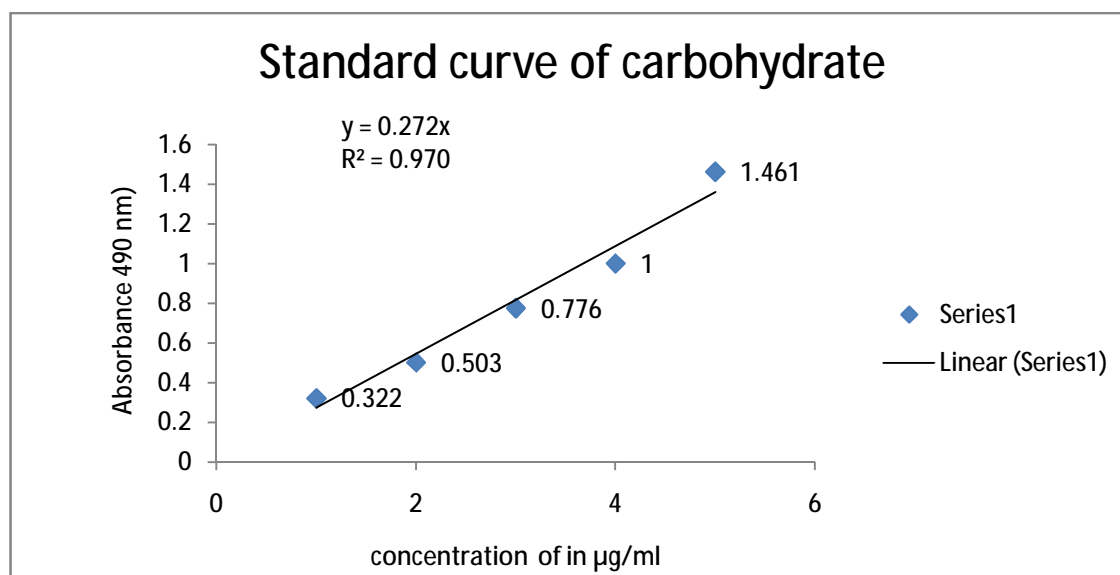


Fig.13 Standard curve of glucose

Table no.9. Calculated amount of carbohydrates in EPS with five different sugars

Sugars	Amount in mg/ml (µg)
Glucose	81.9
Fructose	70.45
Sucrose	78.8
Mannitol	135
Lactose	107.5

- From the above table it was found that the sugar concentration is maximum in the EPS that was extracted from.
- Hence we can say that the medium with is better source to extract EPS that will confer greater sugar concentration.
- Carbohydrates are important for attachment, sometimes in locomotion and dessication resistance.

### B. Protein estimation

#### Standard curve for proteins:

Table no.10. Observation table with BSA as standard protein

BSA( $\mu$ l)	PBS( $\mu$ l)	Bradford reagent (ml)	O.D.(595 nm)			Mean
2	98	5	0.075	0.023	0.041	0.046
4	96	5	0.061	0.079	0.072	0.070
6	94	5	0.141	0.207	0.173	0.173
8	92	5	0.134	0.319	0.345	0.266
10	90	5	0.525	0.236	0.398	0.386

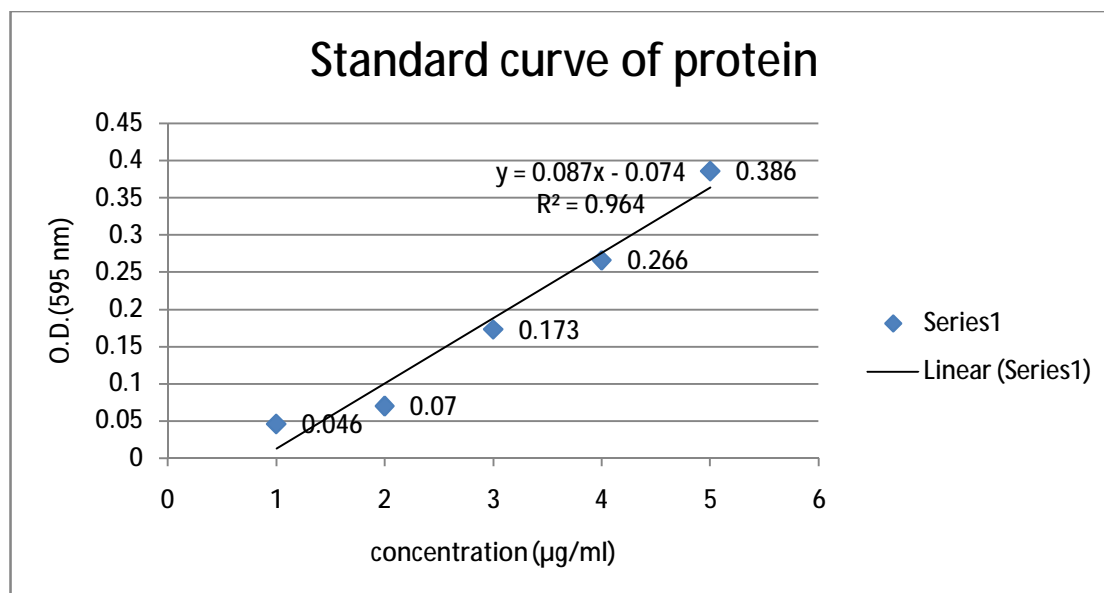


Fig.14 Standard curve with BSA as standard protein



Table no.11. **Calculated amount of protein in EPS with five different sugars**

<b>Sugars</b>	<b>Amount in 1mg/ml (µg)</b>
Glucose	49
Fructose	68.75
Sucrose	123
Mannitol	30
Lactose	48

- So we found that the maximum amount of protein occurs in the EPS which is extracted from the sugar.
- Hence to obtain a greater proteinaceous property sugar medium will be beneficial.
- Protein can contribute to hydrophobicity which is an important factor in bioflocculation of cells.



## 7. FTIR ANALYSIS

### a) *With mannitol as carbon source*

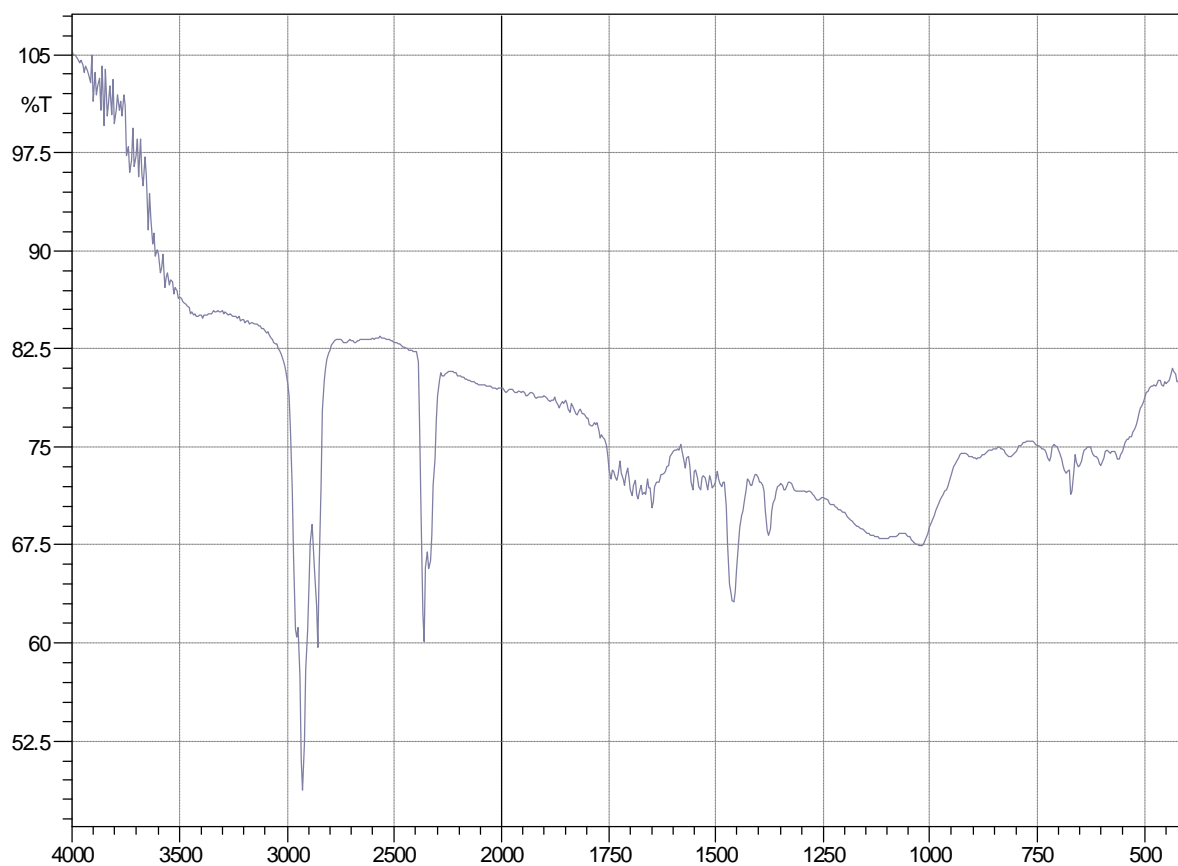
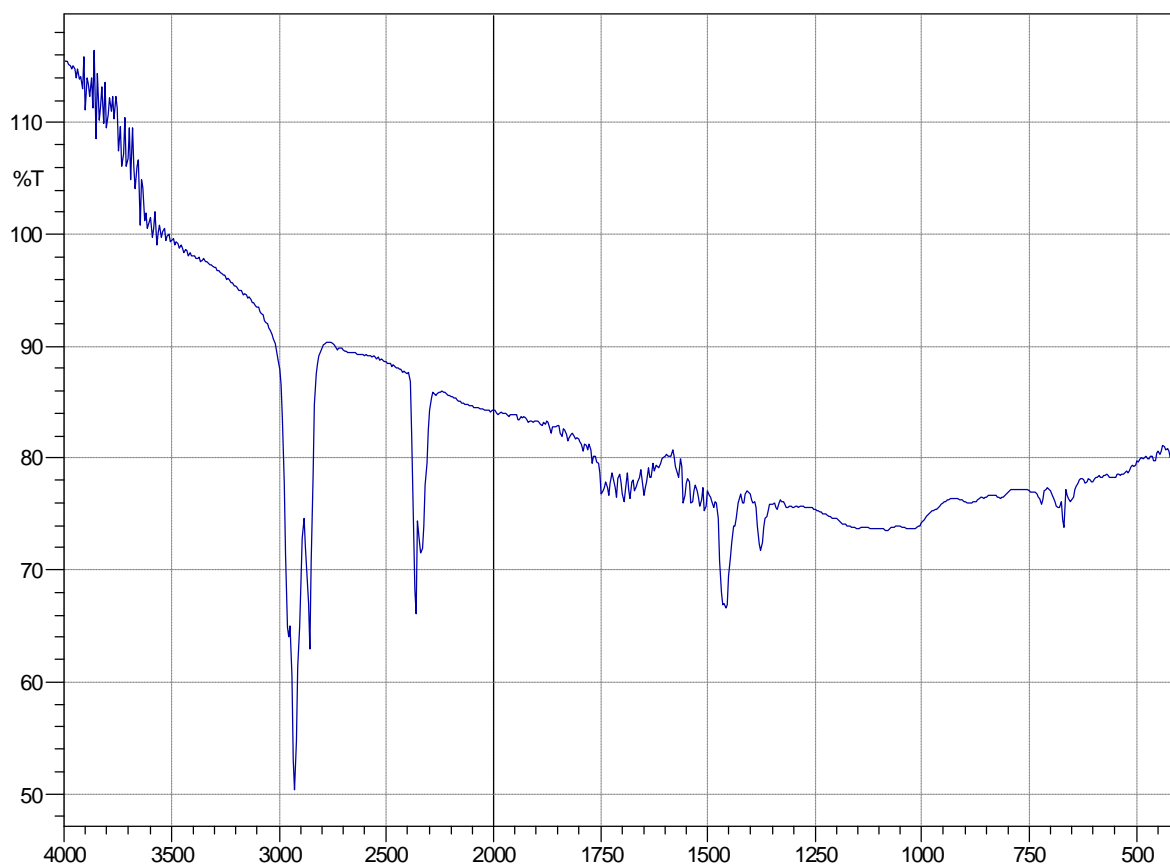


Fig.15 FTIR analysis of compound extracted in fructose

Table no.12 Functional groups corresponding to peaks (in fructose)

2900	C-H	Alkanes
2800	C-H	Alkanes
2400	O-H	Carboxylic group
1450	CH	Alkanes
1300	C-N	Amines
1000	C-O	Alcohols
700	C-H	Alkynes

***b) With sucrose as carbon source***



**Fig.16 FTIR analysis of compounds extracted with sucrose**

**Table no.13 Functional groups corresponding to peaks (in sucrose)**

2900	C-H	Alkanes
2800	C-H	Alkanes
2400	O-H	Carboxylic group
1400	C-F	Alkyl halides
1350	S=O	Sulphonates
650	C-H	Alkynes

## **DISCUSSION**

Studies earlier have shown that to obtain an accurate count we need to 20-200 colonies per plate. Plates that fall outside this range won't give accurate counts. In this experiment the sample was diluted up to  $10^{-4}$  dilution. Spreading was done using  $10^{-3}$  and  $10^{-4}$  sample. And we got a colony count of 68 in the  $10^{-3}$  and a count of 40 in  $10^{-4}$  dilution. Hence the counting can be accurately done. There are studies stating that slow growing *Rhizobium* bacteria need an incubation of 3-5 days to form white, glistening and smooth colonies, while the fast growing ones can be developed in 1-3 days. The growth of *Rhizobium* colony appeared within the three days incubation. And the colony developed as round, white and mucoid (slimy) colonies.

Generally the *Rhizobium* is Gram negative bacillus. After the bacteria was isolated from the root nodules it was subjected to Gram staining. It appeared pink and rod like in shape, hence it was taken to be as Gram negative and bacillus. It was also stated that in the YEM agar plus congo red medium, it tends to absorb the color of the dye. Even our culture appears pinkish when it is grown in congo red plus YEM agar media because it has absorbed the dye.

Using the Hi-carbo kit we generally visualize the ability of the microorganisms to solublize different sugars. In our experiment, the culture shows positive results towards the sugars like lactose, fructose, maltose, xylose, dextrose, galactose, trehalose, mellibiose, sucrose, L-arabinose, mannose, glycerol, adenitol and esculin hydrolysis since we get a colour change in those compounds. This means that the culture is able to solublize the given compounds. While for the rest sugars in the kit shows negative results.

Mannitol is used in the YEM agar media as sugar (carbon) source. So four different sugars like glucose, fructose, sucrose and lactose were used separately in the media to extract EPS. Prior to the addition of those sugars in the media these need to be filtered through syringe filter. This filtration is done because the direct addition of sugar would result in the charring of the media. Once the media with different sugars are prepared and the culture is inoculated, it kept for three days incubation which is a required period for the growth of the bacteria. These are kept in constant shaking so that the culture is thoroughly dispersed.

After the incubation period, the samples are centrifuged so that we can separate the bacterial cells from the supernatant. This supernatant contains the substances released by microorganisms. The EPS were extracted with five different sugars using phenol precipitation

method. Generally twice the volume of chilled ethanol was used to precipitate. To prepare a standard curve of carbohydrate, glucose is taken as the standard sugar so that we can compare our samples absorbance with that of the standard curve. The total carbohydrate content in EPS is calculated by matching the absorbance with the concentration. Now when we compare the values, we obtain different amounts in different sugars. The maximum sugar was found in the EPS extracted from mannitol medium and minimum was obtained from fructose. Similarly for estimation of protein in the EPS samples, we prepare a standard curve of protein using BSA as the standard. On comparing the amount of protein obtained in our samples, we get maximum amount in sucrose medium while lesser amount is found in mannitol.

The FTIR analysis helps to find the different functional groups present in the samples. It is a technique which is used to get an infrared spectrum of emission, absorption, photoconductivity or scattering of a matter. An FTIR spectrometer is known to collect spectral data in a wide range. This confers a significant advantage over a dispersive spectrometer which measures intensity over a narrow range of wavelengths at a time. Our EPS sample was taken for FTIR analysis to determine the functional groups that ultimately gives the idea of different substances present in the sample. For instance we got different peaks for the samples using different sugars. The maximum peaks were obtained in the range of 2400 and 2900. Peaks were also found in the range of 600 to 1750.

For the EPS extracted in mannitol

- peaks at 2900 and 2800 marked the presence of alkanes.
- Peak at 2400 stands for carboxylic group
- At 1450 alkanes are present.
- At 1300 amines are present.
- Peaks at 1000 and 700 signifies alcohols and alkynes respectively.

For the EPS extracted in sucrose three different peaks were obtained along with the peaks at 2900, 2800 and 2400.

- Peaks at 2900 and 2800 signified presence of alkanes.
- The peak at 1400 signifies the presence of alkyl halides.
- At 1350 sulphonates are present.
- At 650 alkynes are present.

- Comparing the peaks and values it was found that majority of functional groups were those which are present in carbohydrates. Rest of the peaks also signified some functional group related to proteins, amino acids and sugar acids.

A greater amount of carbohydrates will help bacteria to attach to different surfaces. It will also help in the locomotion and in tolerating extreme dry conditions. A good protein content will help in providing hydrophobicity in bio-flocculation of cells, through which it can clump and settle the organic solids in water.

## **CONCLUSION**

The root nodules were found to occur after four weeks of planting the legume plant, *Vigna radiata*. The root nodules were round to pinkish yellow in appearance, which formed due to the symbiotic association of the soil bacteria, *Rhizobium*. The bacteria as *Rhizobium* was identified by the growth characteristics in the Yeast extract mannitol agar (YEMA) media and then adding the media with congo red agar and with Gram staining. In the simple YEM agar media it showed white round slimy type of colonies and in media where Congo red was added, it tend to absorb the colour and appeared white. The general incubation period was of three days which concludes that the bacteria is a slow growing bacteria.

The bacteria produced a white slimy colony. This slimy nature was due to the production of the extracellular polymeric substance (EPS). The EPS secreted by the bacteria was extracted by ethanol precipitation method and analyzed biochemically. The EPS was tested for carbohydrates and proteins. It was found that with different carbon sources i.e the medium containing different sugars like glucose, fructose, sucrose, mannitol and lactose, the composition of EPS varied. Then the standard curve of sugars was drawn following the Phenol-sulphuric method, taking glucose as standard. Similarly prior to the protein estimation, a standard curve was plotted by Bradford reagent method. Then taking the extracted EPS, the carbohydrate and protein estimation was done. Out of the five sugars used the maximum sugar percentage was found, when it was grown in Mannitol medium. Similarly the greater protein content was found when EPS was extracted with Sucrose medium. Thus we can conclude that for EPS to have maximum carbohydrates, the mannitol medium is suitable while the sucrose medium is suitable for protein content.

After the FTIR analysis, it was confirmed that major functional groups belonged to alkanes, carboxylic groups and some traces of amines, suphonates and alcohols, which signifies that majority of components of EPS are the carbohydrates, some amount of proteins and sugar acids like the uronic acids and also traces of nucleic acids. It also signifies that change in the functional groups was because of the different sugar sources used. The change in the composition of EPS with regard to its functional groups will also results in the change in the properties.

Hence the EPS with greater number of functional groups seen in carbohydrates will be having the majority of carbohydrates. So it will also be seen in case of the proteins which will correspond to its functional groups.

As far as its application is concerned, the EPS with more sugar content will have better attachment to the surfaces. In addition to that it will also serve for its locomotion and desiccation resistance. This helps the microorganisms to withstand or endure extreme dryness or drought like conditions. And the protein will be responsible for providing the hydrophobicity that in turn will be accounting for bio-flocculation of cells. This bio-flocculation helps in the clumping of dispersed organic substances that result in the more efficient settling of organic solid products in water.

The EPS have the ability to bind with heavy metals from different environments. They have roles in the degradation process of organic materials, the denitrification of waste products, elimination of phosphate ions from manufacturing and municipal wastes. In the food industries, the novel exopolysaccharides may bring improvement in the cost, hydration of food products, and production of low calorie foods. For food edible coatings can be also applied using the extracellular polysaccharides to protect food from spoilage. Removal of attached bacteria from the surface can also be targeted through a better understanding of the factors affecting the biosynthesis of EPS. This may create new prospective for the environmental use of the microorganisms.

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